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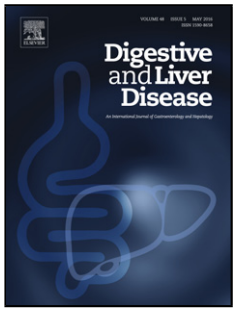

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GLUTEN-FREE DIET DOES NOT INFLUENCE THE OCCURRENCE AND THE TH1/TH17-TH2 NATURE OF IMMUNE-MEDIATED DISEASES IN PATIENTS WITH COELIAC DISEASE

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Matilde Rea: data collection

Nicola Caporaso: approval of the final version of the manuscript

Raffaella Tortora: substantial contribution to conception and design of the study, drafting the article

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SUMMARY

Introduction: Coeliac disease (CD) is the most common Th1-mediated enteropathy, frequently associated with other immune-mediated disorders (IMD). **Aims:** to evaluate: 1) the prevalence of IMD at the time of and after CD diagnosis; 2) a possible change in immune response to gluten free diet (GFD); 3) the potential role of GFD in reducing and/or preventing IMD in CD. **Methods:** prospective study including all consecutive adult CD patients who underwent investigations for Th1-Th17/Th2-IMD at the time of CD diagnosis and after a 5-year follow-up period. **Results**: 1255 CD were enrolled. Of these, 257 patients (20.5%) showed IMD at the time of CD diagnosis, with 58.4% presenting a Th1/Th17-IMD. After a 5-year follow-up period, 682 patients (54.3%) showed new IMD despite GFD. Of these, 57.3% presented a Th1/Th17-IMD and 42.7% a Th2-IMD (p=0.8). When compared the prevalence of each type of IMD before and after CD diagnosis, we did not identify any significant "switch" from Th1/Th17- to Th2-IMD or vice versa. The number of patients with Th1/Th17- and/or Th2-IMD increased during the GFD period (20.5% vs 54.3%; p<0.01; OR 1.9). **Conclusions:** The prevalence of IMD at the time of CD diagnosis is high and it seems to increase in the follow-up period despite GFD.

KEY WORDS*: Coeliac disease, gluten, autoimmunity, immuno-mediated diseases, Th1, Th2, Th17*

INTRODUCTION

Coeliac disease (CD) is the most common enteropathy in Western genetically susceptible subjects (HLA DQ2/DQ8) **[1]** mediated by a T helper cell type (Th) 1 immune response to gluten, a complex of water insoluble proteins from wheat, barley and rye **[2]**.

Indeed, as a chronic autoimmune disorder, both innate and adaptive immune responses are involved in the pathogenesis of CD **[3].**

Some studies have shown how the translocation of α2-gliadin-33mer may depend on an apical-basal transcytosis stimulated by INF-γ, a cytokine involved in immunopathogenesis of CD **[4]**. Once it has reached the lamina propria, the gliadin would react with the tissue transglutaminase (tTG) – i.e. the enzyme catalyzing glutamine's deamidation– thus creating a transglutaminase-gliadin deamidated complex **[5].** The deamidated peptide would be picked up by HLA DQ2 or DQ8 molecules on the surface of antigen-presenting cells (APC), and would be "presented" to the CD4 + T helper 1 (Th1). These Th1-cells would produce high levels of pro-inflammatory cytokines (IL2, IL6, INFγ, TNF), which could promote an increased cytotoxicity of intraepithelial lymphocytes (IELs) and natural killer (NK) T cells, causing apoptosis of enterocytes and the production of Th2 cytokines activating B cells and favoring the differentiation of plasma cells, hence resulting in the release of antibodies: anti-gliadin and anti-transglutaminase **[6 – 9].**

Despite this pathogenesis, it seems that both Th1/Th17- and Th2-mediated diseases may co-exist in cases of CD **[10].** It is well known that CD patients show a particular tendency for multiple autoimmune and/or allergic and/or immuno-mediated disorders (IMD) over their lifetime **[11-12],** which supports this possibility.

To date, only hypotheses exist to explain the observed split in the association between the type of T lymphocyte-mediated reaction and CD. CD is frequently associated to other Th1/Th17-IMD

[13-15], such as type 1 diabetes mellitus **[16]**, autoimmune thyroiditis **[17]**, rheumatoid arthritis **[18]**, psoriasis **[19]**, multiple sclerosis **[20]**. More recently, several studies have also shown an association between CD and some Th2-mediated disorders **[15]**: allergies and asthma, eczema, rhinitis **[21]**, urticaria **[22],** Grave's disease **[17]**, Sjogren syndrome **[23]**, lichen planus **[24]**, systemic lupus erythematosus **[25].**

To date, a gluten free diet (GFD) is considered the only treatment for CD. Numerous papers have investigated the effects of CD therapy on the incidence and prognosis of coexisting or subsequent IMD but thus far they have reported contradictory results **[26-35].** The aims of our study were: 1) to establish the prevalence of IMD at the time and after CD diagnosis in a large sample of adult individuals; 2) to identify any possible changes in immune response after commencement of GFD, in particular with regard to shifts from Th1/Th17- to Th2-immune response or vice versa; and 3) to investigate the potential role of GFD in reducing and/or preventing IMD in adult CD patients.

METHODS

Between September 2011 and February 2015 we carried out a prospective study including all consecutive adult CD patients (age > 18 years) followed up at our Gastrointestinal Unit (Tertiary Centre for Food Intolerance and CD, "Federico II" University, Naples, Italy). In accordance with current guidelines, CD diagnosis was made in the presence of Marsh ≥ 2 histology associated with both anti-tissue Transglutaminase (a-tTG) IgA > 7 U/ml and positive anti-endomysial (EMA) antibodies **[11]**. All subjects with positive serology but negative histology (Marsh 0 or 1) underwent further investigations for genetic susceptibility and were diagnosed as "potential" CD in the presence of HLA DQ2/DQ8. The main demographic, clinical, serological, endoscopic and histological features were recorded for all CD patients. All patients were investigated for the presence of Th1/Th17 and/or Th2-IMD at the time of CD diagnosis. The diagnosis of pre-existent and new IMD was made by the evaluation of medical records with the confirmation of a specialist visit (i.e. rheumatologist, dermatologist, immunologist) and laboratory tests. The classification of Th1/Th17 and Th2-IMD was made in line with current guidance **[13-15].**

Information on the presence/absence of any allergic, atopic, or autoimmune disease preceding CD diagnosis was gathered at the time of the first consultation and during the study period each study participant underwent clinical examination and laboratory tests at least once a year.

In particular, we assessed the clinical and biochemical presence of: vitiligo, allergic rhinitis, ankylosing spondylitis, Sjogren syndrome, multiple sclerosis, scleroderma, nephrotic syndrome, ulcerative colitis, psoriasis, polymyalgia rheumatica, urticaria, IgA nephropathy, lichen planus, systemic lupus erythematosus, type 1 diabetes mellitus (or latent autoimmune diabetes in adults), Hashimoto's thyroiditis, eczema, atopic dermatitis, primary sclerosing cholangitis, Crohn's disease, allergic conjunctivitis, primary biliary cirrhosis, Grave's disease, asthma, alopecia, rheumatoid arthritis and other allergies. Table 1 summarizes the main Th1-, Th17- and Th2-

IMD. The search for Th1/Th17 and/or Th2-IMD was reassessed after starting GFD in a 5-years follow-up period.

Statistical analysis

Data were analyzed using the Statistical Package for Social Sciences (SPSS software v.15.0, Chicago IL, United States) for Windows. The descriptive statistics included determination of mean values and standard deviation (SD) of the continuous variables, and the percentages and proportions of the categorical variables. The chi-square (χ^2) test was used to assess the comparison of categorical variables, while the Mann-Whitney U test was used for unpaired data. Analysis of variance (ANOVA) was performed with and without adjustment for covariates. The odd ratio (OR) for quantifying the statistical difference between the dichotomous variables was also calculated. All results were considered statistically significant when $p\ value < 0.05$.

RESULTS

During the study period, 1255 CD patients referred to our Centre were enrolled (males/females 258/997). At the time of CD diagnosis, mean age ± SD was 28,1 ± 15,7; mean a-tTG serum level was 98,7 ± 108,2 U/mL; all patients were positive for EMA. Histological exam showed a Marsh 1 grade in 64 patients (5%), Marsh 2 grade in 50 (4%), Marsh 3A in 171 (13.7%), Marsh 3B in 311 (24.8%), and Marsh 3C in 659 (52.5%). Main patient characteristics and diagnostic results are reported in Table 2.

Data collected through medical history showed that 257 patients out of 1255 (20.5%) suffered from at least one immunological/allergic IMD before the diagnosis of CD. Of these, 150 (58.4%) suffered from Th1/Th17-mediated and 107 (41.6%) from Th2-mediated diseases (p=0.7). Table 3 reports the prevalence of all IMD found in our population.

In the 5-years study period following CD diagnosis, 682 patients out of 1255 (54.3%) developed other immuno-mediated diseases regardless of whether they were following a strict GFD (mean a-tTG levels were 1,2 ± 0,8 U/mL; negative EMA). Of these 682 patients, 391 (57.3%) developed a Th1/Th17-related disease, and 291 (42.7%) a Th2-mediated condition (p=0.8). Also in this case, Table 3 summarizes all results relating to the type of IMD present and their prevalence at the time of diagnosis and after GFD.

We compared the prevalence of immuno-mediated diseases pre- and post-CD diagnosis and found that there was no significant 'switch' from Th1/Th17-mediated to Th2-mediated response or vice versa (58.4% and 41.6%, respectively, pre-CD diagnosis; 57.3% and 42.7% post-CD diagnosis; p=0.9). The number of patients with either a Th1- or a Th2-mediated disease increased over time in the period following CD diagnosis (20.5% vs 54.3%; p<0.01; OR 1.9;95% C.I. 1.5-2.1). Of the 1255 participants in the study, 150 (12%) were affected by Th1/Th17-IMD at the time of CD diagnosis and 391 (31%) following CD diagnosis (p<0.01; OR 2.6); 107

(8.5%) showed a Th2-IMD at the time of CD diagnosis and 291 (23.3%) following CD diagnosis ($p<0.01$; OR 1.6; 95%C.I. 1.3-1.9).

When we examined the prevalence of the most frequent CD-related IMD, we found that Hashimoto's thyroiditis was present in 8.2% of patients at the time of CD diagnosis and in 24% of patients after CD diagnosis ($p<0.01$; OR 1.6, 95%C.I. 1.5-1.7); 0.7% of patients suffered from psoriasis before CD diagnosis while 2.7% developed it after CD diagnosis ($p<0.01$; OR 1.5; 95%C.I. 1.3-1.8); type 1 diabetes mellitus was present in 1.8% of patients at the time of CD diagnosis and in only 0.2% after CD diagnosis ($p<0.01$; OR 0.08, 95%C.I. 0.01-0.4) (Table 3).

No correlation was found between the occurrence of autoimmune/allergic diseases and any of the following variables: age at the time of CD diagnosis, a-tTG serum levels at the time of diagnosis and at follow-up, Marsh grade and clinical symptoms (P = NS). Table 4 better clarifies these results.

DISCUSSION

At present, the only treatment for CD is represented by gluten free diet (GFD).

Numerous papers have explored the effects of GFD on the incidence and course of CD-associated IMD, often reporting contradictory results. Most of these studies, however, supported the hypothesis of a protective effect of GFD with regard to the occurrence of CD-associated IMD **[12].**

Our study investigated the relationship between GFD and CD-associated IMD with a specific focus on the relative prevalence of Th1/Th17- and Th2-mediated diseases before and after the start of GFD. Our results suggest that we should re-examine the widely shared view that GFD is a magic bullet for the treatment of all pathological conditions associated with CD. Our study included a large population of patients suffering from CD (1255 in total), which strengthens our results and suggestions for practice. The present study confirms the outcomes firstly reported by Elli et al **[30 – 31]**. In particular, the Authors explored the rate of IMD in 1015 CD patients reporting a significantly elevated prevalence (23%) of IMD in CD. Interestingly, the occurrence of IMD was not influenced by sex, GFD compliance, and time of gluten exposure.

On the other hands, in a French multicentric study on adult and pediatric CD patients, Cosnes et al **[26]** retrospectively evaluated 927 coeliac patients. Of these, 178 (19.3%) developed one or several autoimmune diseases. The cumulative risk of autoimmune disease was 8.1% $\pm$ 1% at age 15, and 15.7% $\pm$ 1.5% at age 30. The cumulative risk of subsequent autoimmune disease was lower in patients who followed a GFD than in those who did not ($p<0.05$). The incidence of autoimmune disease was 5.4/1000 patient-years in the case of adherence to a GFD vs 11.3/1000 patient-years in the case of non-adherence to the diet ($p < 0.002$). The Authors concluded that factors associated with an increased risk for immuno-mediated diseases were family history of autoimmunity (hazard ratio 2.36) and diagnosis of coeliac disease before 36 years of age (hazard ratio 2.65). In other

words, for Cosnes and colleagues GFD seemed to have a protective role, maybe by contributing to re-establishing the integrity of the intestinal barrier. The suggestion by Cosnes et al., however, contrasted with the results from another large Italian study by Ventura et al. **[29]** (carried out with a sample population of 909 coeliac patients) which concluded that a higher prevalence of other autoimmune disorders was associated with longer duration of exposure to gluten.

Some studies exist which question the protective effect of GFD on the occurrence of CD-associated IMD. In 2005, Viljamaa et al **[27]** studied 703 CD patients: even though they found an increased risk of autoimmune disease in CD patients (prevalence of 21.8%), the Authors concluded that the duration of gluten exposure seemed not to be of crucial importance in the development of autoimmune diseases. A similar conclusion was reached by Sategna Guidetti et al. **[37]**, who reported a prevalence of autoimmune disease in CD patients of about 30%. In our study, we did not find any correlation between the development of IMD and the age at the time of diagnosis. It seems to us that, once started, the "autoimmune waterfall", cannot be stopped by GFD, thus leading to an increased risk of developing IMD. About this issue, a different conclusion was reached by Elli et al **[30]** reporting a quite protective effect of longstanding gluten exposure on the occurrence of IMD. In effect, the Authors found that the increment of 1 year in CD diagnosis corresponded to a lower risk of developing IMD (HR 0.30).

With regard to the CD-related IMD found in our study population, the most frequent were: Hashimoto's thyroiditis (8.2% before vs 24% after CD diagnosis; $p<0.01$; OR 1.6 (IC:1.5-1.7)); psoriasis (0.7% before vs 2.7% after CD diagnosis; $p<0.01$; OR 1.5 (IC 1.3-1.8)); type 1 diabetes mellitus (1.8% before vs 0.2% after CD diagnosis; $p<0.01$; OR 0.08 (IC 0.01-0.4)).

The association between CD and thyroid disorders is well known. In a study conducted by Hadithi et al. **[38],** 4.8% of Dutch patients with Hashimoto's thyroiditis had CD, and 12% of patients with CD had Hashimoto's thyroiditis. In a prospective study of 27 adult coeliac patients, Metso et al. **[32]** found that CD subjects had an elevated anti-TPO levels, which continued to increase despite GFD. Thyroid atrophy also progressed while on a GFD. The Authors suggested that GFD seemed

not to prevent the progression of the autoimmune process in adults during a 1-year follow up. The observation by Metso et al. is in accordance with the results from our study: 24% of CD patients showed Hashimoto's thyroiditis during the study period but only 8.2% of patients had presented thyroiditis before CD diagnosis ($p<0.01$; OR 1.6).

The association between psoriasis and CD remains unclear **[39 – 40].** Ludvigsson et al. **[41]** found that CD was a risk factor for future psoriasis (hazard ratio 1.72). In our study population, 0.7% of patients suffered from psoriasis before CD diagnosis while 2.7% developed it during the post-CD diagnosis period ($p<0.01$; OR 1.5), confirming previous results.

Several recent studies have showed a higher prevalence of Metabolic Syndrome (MS) and its components among subjects suffering from psoriasis (between 4.3 and 40%), compared to the general population. A recent systematic and meta-analytical review of several observation studies described an OR of 2.26 for MS in subjects with psoriasis **[42].** Psoriasis appears more frequent in obese subjects **[43].** In a recent publication, our team **[44]** demonstrated a high risk for development of MS in CD patients on GFD. This finding could explain an important common pathogenetic mechanism linking CD and psoriasis.

The association between CD and type 1 diabetes mellitus is well established. In the majority of cases (more than 90%), the diagnosis of diabetes precedes that of CD, as confirmed by Greco et al. **[45]**. However, some studies have reported that diabetes onset can frequently occur in patients already diagnosed with CD. Valerio et al. **[46]**, for example, demonstrated that diagnosis of CD prior to onset of diabetes denotes a subgroup of patients with a more severe clinical presentation of diabetes and higher prevalence of multiple autoimmune diseases, suggesting that GFD does not help to prevent other autoimmune disorders which have already been triggered. In our study population diabetes was present in 1.8% of patients before CD diagnosis, but after starting GFD only 0.2% presented diabetes ($p<0.01$; OR 0.08). This result could be explained by the fact that ours is an adult population. In effect, type 1 diabetes mellitus usually presents in adolescent and young adults, except for latent autoimmune diabetes of the adults (LADA) which affected 2 patients in the study.

In our paper we consider also the Th2-mediated diseases, such as allergic diseases. No statistically significant differences were found between the percentage of Th2-predominant disorders before and after CD diagnosis (41.6% and 42.7%, respectively, P = NS), although the absolute number of patients affected by a Th2 disease increased during GFD (107 vs 291, $p < 0.01$). Therefore, it seems that the coeliac population is also particularly susceptible to allergic diseases, even after starting GFD.

However, there were some limitations to our study. In this paper, we used a-tTg as a surrogate marker for GFD adherence at 5-year follow-up. This method used to assess adherence has limitations, and may not accurately reflect true adherence/mucosal healing **[47],** even though current guidelines suggest to perform upper endoscopy with intestinal biopsies in cases with lack of clinical response or relapse of symptoms despite a GFD **[2].** Furthermore, we had no data on IMD occurrence in non-CD patients. However, a previous paper by Elli and colleagues **[30]**, comparing the prevalence of IMD in 1015 CD patients with those of a registry population (848.606 subjects), underlined a significantly high percentage of IMD in CD patients respect to the controls (23% vs 0.4%); these results confirm the specific role of CD in predisposing patients to other IMD.

In summary, CD has been associated with different IMD and allergic diseases, especially Hashimoto's thyroiditis, type 1 diabetes mellitus and psoriasis. The prevalence of immune-mediated diseases at the time of CD diagnosis is high and it seems to increase in the follow-up period. GFD does not influence (and in particular, it does not reduce) the prevalence, the occurrence or the Th1/Th17-Th2 nature of immune-mediated diseases in CD. Our results therefore suggest we should re-examine the widely shared view that GFD is a sort of magic bullet for all pathological conditions associated with CD.

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Table 1. Classification of main Th1/Th17 and Th-2 mediated disorders.

Th1/Th17-mediated diseases	Th2-mediated diseases
Coeliac disease	IgE related-allergies
Vitiligo	Sjogren syndrome
Ankylosing spondylitis	Allergic conjunctivitis
Multiple sclerosis	Atopic dermatitis
Nephrotic syndrome	Grave's disease
Crohn's disease	Ulcerative colitis
Psoriasis	Asthma
Polymyalgia rheumatic	Urticaria
Alopecia	Eczema
IgA nephropathy	Scleroderma
type 1 Diabetes Mellitus	Systemic lupus erythematosus
Hashimoto's thyroiditis	Allergic rhinitis
Rheumatoid arthritis	Lichen planus
Primary biliary cirrhosis	Other allergies
Primary sclerosing cholangitis	

Table 2. Main patient characteristics and diagnostic results of 1255 coeliac patients.

		Number (%)
Demographic data	Male	258 (20.6)
	Female	997 (79.4)
	Mean age ± SD	28.1 ± 15.7
Symptoms (in accordance with Oslo) [11]	Classical CD	454 (36.2)
	Non Classical CD	627 (49.9)
	Asymptomatic CD	110 (8.7)
	Potential CD	64 (5)
Risk factors for CD	Down Syndrome	8 (0.6)
	Familial history of CD	406 (32.3)
Laboratory	a-tTG (U/ml)	98.7 ± 108.2
	EMA positive	100%
Histology	Marsh 1	64 (5)
	Marsh 2	50 (4)
	Marsh 3A	171 (13.7)
	Marsh 3B	311 (24.8)
	Marsh 3C	659 (52.5)

Table 3. Main immune-mediated diseases presenting before (Pre) and after (Post) CD diagnosis. They were divided into Th1/Th17 and Th2-mediated conditions; *p* is expressed only in case of statistically significant results.

Th1/Th17-Th2	Disease	Pre * n° (%)	Post ** n°(%)	*p*	OR
Th1/Th17 mediated conditions	Vitiligo	4 (0.3)	7 (0.5)	0.3	
	Ankylosing spondylitis	-	4 (0.3)	0.1	
	Multiple sclerosis	-	1 (0.1)	0.9	
	Nephrotic syndrome	-	3 (0.2)	0.3	
	Crohn's disease	-	7 (0.5)	0.09	
	Psoriasis	9 (0.7)	35 (2.8)	0.01	1.5
	Polymyalgia rheumatic	1 (0.1)	3 (0.2)	0.3	
	Alopecia	7 (0.5)	8 (0.6)	0.7	
	IgA nephropathy	-	1 (0.1)	0.9	
	type 1 dabetes mellitus	22 (1.8)	2 (0.2)	<0.01	0.08
	Hashimoto's thyroiditis	103 (8.2)	301 (24)	<0.01	1.6
	Rheumatoid arthritis	4 (0.3)	15 (1.2)	0.09	
	Primary biliary cirrhosis	-	2 (0.2)	0.5	
	Primary sclerosing cholangitis	-	2 (0.2)	0.5	
Th2 mediated conditions	Ulcerative colitis	-	15 (1.2)	0.08	
	Allergic conjunctivitis	1 (0.1)	11 (0.9)	0.1	
	Atopic dermatitis	8 (0.6)	26 (2.1)	0.08	
	Grave's disease	4 (0.3)	20 (1.6)	0.1	
	Asthma	33 (2.7)	47 (3.8)	0.1	
	Urticaria	8 (0.6)	24 (1.9)	0.2	
	Eczema	1 (0.1)	11 (0.9)	0.3	
	Scleroderma	-	10 (0.8)	0.1	
	Systemic lupus erythematosus	1 (0.1)	13 (1)	0.09	
	Allergic rhinitis	35 (2.8)	53 (4.2)	0.08	
	Lichen planus	-	11 (0.9)	0.1	
	Sjogren syndrome	-	13 (1)	0.09	
	Other allergies	16 (1.3)	37 (2.9)	0.01	
		257 (20.5)	**682 (54.3)**	**<0.01**	**1.9**

* All IMD diagnosed before CD diagnosis
** All new diagnoses of IMD found after CD diagnosis during the follow-up

Table 4. Correlation between the occurrence of IMD and: age at the time of CD diagnosis, a-tTG serum levels at the time of diagnosis and at follow-up, clinical symptoms, Marsh grade.

		IMD	No IMD	*p*
Age (years) at time of CD diagnosis (mean ± SD)		29.93 ± 16.66	30.93 ± 14.03	0.2
tTG IgA levels at time of CD diagnosis (U/mL) (mean ± SD)		89.73 ± 90.95	93.84 ± 85.88	0.4
tTG IgA levels at follow-up (U/mL) (mean ± SD)		1.2 ± 0.9	1.3 ± 1.2	0.09
Symptoms (in accordance with Oslo) [11]	**Classical CD (%)**	56.4%	43.6%	0.1
	Non Classical CD (%)	55.9%	44.1%	0.2
	Asymptomatic CD (%)	56.3%	43.7%	0.1
Marsh Grade	**Marsh 1 (%)**	5.1%	5%	0.9
	Marsh 2 (%)	3.8%	4.2%	0.7
	Marsh 3 (%)	91.1%	90.8%	0.7